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Examination of fungal stress response genes using *Saccharomyces cerevisiae* as a model system: targeting genes affecting aflatoxin biosynthesis by *Aspergillus flavus* Link

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Abstract *Saccharomyces cerevisiae* served as a model fungal system to examine functional genomics of oxidative stress responses and reactions to test antioxidant compounds. Twenty-two strains of *S. cerevisiae*, including a broad spectrum of singular gene deletion mutants, were exposed to hydrogen peroxide (H_2O_2) to examine phenotypic response to oxidative stress. Responses of particular mutants treated with gallic, tannic or caffeic acids, or methyl gallate, during H_2O_2 exposure, indicated that these compounds alleviated oxidative stress. These compounds are also potent inhibitors of aflatoxin biosynthesis in *Aspergillus flavus*. To gain further insights into a potential link between oxidative stress and aflatoxin biosynthesis, 43 orthologs of *S. cerevisiae* genes involved in gene regulation, signal transduction (e.g., *SHO1*, *HOG1*, etc.) and antioxidation (e.g., *CTT1*, *CTA1*, etc.) were identified in an *A. flavus* expressed sequence tag library. A successful exemplary functional complementation of an antioxidative stress gene from *A. flavus*, mitochondrial superoxide dismutase (*sodA*), in a *sod2*Δ yeast mutant further supported the potential of *S. cerevisiae* deletion mutants to serve as a model system to study *A. flavus*. Use of this system to further examine functional genomics of oxidative stress in aflatoxigenesis and reduction of aflatoxin biosynthesis by antioxidants is discussed.

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Introduction

The genome of *Saccharomyces cerevisiae* Meyen ex. E. C. Hansen has been sequenced and well annotated. Strains of *S. cerevisiae* having individual open reading frames functionally deleted (Winzeler et al. 1999) can be used to identify gene-targets of chemicals or drugs (Tucker and Fields 2004) or stress, such as oxidative stress, response pathways (Toone and Jones 1998). Such singular deletion mutants of *S. cerevisiae* might also serve to gain a better understanding of how oxidative stress affects mycotoxin biosynthesis in relatively genetically uncharacterized fungi, such as aflatoxin by *Aspergillus flavus* Link. Aflatoxins are hepato-carcinogenic difuranocoumarins, produced most notably by *A. flavus* and *A. parasiticus* Speare, which can contaminate a number of agricultural commodities, affecting food safety and agricultural trade (Campbell et al. 2003). Understanding the molecular mechanisms regulating aflatoxin biosynthesis may provide valuable insights on how to inhibit its biosynthesis or that of other mycotoxins (Bhatnagar et al. 2002; Sweeney and Dobson 1999; Yu et al. 2004a).

Gallic acid from hydrolysable tannins arrests aflatoxin biosynthesis (Mahoney and Molyneux 2004) and disrupts expression of early and late aflatoxin biosynthetic pathway genes, such as *nor1* and *ver1*, without affecting transcription of the positive aflatoxin pathway gene regulator, *aflR* (Cary et al. 2003). Hydrolysable tannins and gallic acid are known biological antioxidants (Sroka and Cisowski 2003), and gallotannins can prevent cell death under oxidative stress (Ying and Swanson 2000; Ying et al. 2001). Since oxidative stress triggers aflatoxin biosynthesis in *A. parasiticus* (Jayashree and Subramanyam 2000), the antiaflatoxigenic effect of gallic acid may result from disruption of signal transduction pathway(s) or regulators of *Aspergillus*, such as LaeA (Bok and Keller 2004), triggered by oxidative stress.

Using high-throughput yeast bioassays, we show that gallic acid and related phenolic compounds ameliorate oxidative stress. We also demonstrate functional comple-

mentation by the *A. flavus* antioxidative stress gene, mitochondrial superoxide dismutase (*sodA*), in yeast. Using such tools, the utility of yeast as a model system to further examine stress response-induced biosynthesis of aflatoxin in *Aspergillus* is discussed.

Materials and methods

Microorganisms and culturing conditions

Wild-type BY4741 (*mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and selected deletion mutants of *S. cerevisiae* were obtained from Invitrogen (Carlsbad, Calif.). Deletion mutants and the functions examined are listed in Tables 1 and 2. Yeast culture media included rich medium (YPD; 1% Bacto yeast extract, 2% Bacto peptone, 2% glucose), minimal medium (SG; 6.7 mg ml^{-1}yeast nitrogen base without amino acids, 2% glucose with appropriate supplements: 0.02 mg ml^{-1} uracil, 0.03 mg ml^{-1} amino acids), and complementation medium (SGAL; 6.7 mg ml^{-1} yeast nitrogen base without amino acids, 2% galactose, 0.03 mg ml^{-1} amino acids). Yeast cells were incubated at 30°C without light. *A. flavus* was cultured by inoculating spores (~200) of strain NRRL 25347 into Petri dishes containing VMN agar medium (Vogel 1956). Spore suspensions were prepared by using a cotton swab to transfer spores from fungi grown on potato dextrose agar (Sigma, St. Louis, Mo.) for 7 days at 28°C, to 0.05% Tween 80.

Yeast dilution bioassays

Yeast strains were cultured in YPD liquid medium overnight. Starting with ~1×10^6 cells, five 10-fold serial dilutions were performed using SG liquid medium, resulting in six individual samples from ~10^6 to ~10 cells. Cells from each dilution were spotted adjacently on SG agar plates incorporating the respective test compounds. Nutritional supplements (e.g., amino acids, uracil, etc.) were included in the medium, depending on the experiment. In testing the effects of tannic, gallic and caffeic acids, and methyl gallate on wild-type (WT) cells, oxidative stress was induced by incorporation of 3.3 mM hydrogen peroxide (H_2O_2) into the medium. To examine alleviation of oxidative stress, deletion mutants (and WT) were exposed

Table 1 Responses of wild-type and selected deletion mutants of *Saccharomyces cerevisiae* to oxidative stress induced by exposure to hydrogen peroxide (H_2O_2), and alleviation by antioxidants. Responses are represented as stressed (*S*) if exposure is lethal, and recovered (*R*) if treatment with antioxidants resulted in a decline in lethality. Numbers indicate degree of tolerance to H_2O_2 exposure: *6* cell growth equal to controls having no exposure to H_2O_2, *0* no growth. Decreases are logarithmic (e.g., *1* indicates a 100,000-fold decline in cell growth relative to *6*, etc.). Yeast strains are deletion mutants lacking various genes associated with signal transduction and stress responses. Exemplary bioassays used to construct this table are depicted in Fig. 2

Yeast strain (deletion)[a]	Cellular function	H_2O_2 only	Glutathione	Tannic acid	Gallic acid	Methyl gallate	Caffeic acid
BY4741 (wildtype)	N/A	S3	R 6	R 6	R 6	R 6	R 6
569 (*yap1*Δ)	*jun*-like transcription factor	S 0	R 1	S 0	R 2	R 2	S 0
4718 (*ctt1*Δ)	Catalase T	S 3	R 6	R 6	R 6	R 6	R 6
3615 (*cta1*Δ)	Catalase A	S 3	R 6	R 6	R 5.5	R 6	R 6
2737 (*glr1*Δ)	Glutathione oxidoreductase	S 1.5	R 6	R 2.5	S 1.5	S 1.5	R 2.5
829 (*osr1*Δ)	Glutathione metabolism	S 2.5	R 6	R 6	R 5	R 6	R 6
24190 (*trr1*Δ)	Thioredoxin reductase	S 4	R 6	R 6	R 6	R 6	R 6
1934 (*trr2*Δ)	Thioredoxin reductase	S 3	R 6	R 6	R 6	R 6	R 6
545 (*tsa1*Δ)	Thioredoxin peroxidase	S 2	R 6	R 2.5	S2	R 2.5	R 2.5
4969 (*vph2*Δ)	Assembly of vacuolar H(+)ATPase	S 2	R 6	S2	S 2	S 2	S 2
6681 (*grx1*Δ)	Glutaredoxin	S 2.5	R 6	R 6	R 6	R 6	R 6
4347 (*grx2*Δ)	Glutaredoxin	S 2.5	R 6	R 6	R 5	R 5	R 6
2654 (*trx1*Δ)	Thioredoxin	S 2	R 6	R 5	R 2.5	R 5	R 4.5
4839 (*trx2*Δ)	Thioredoxin	S 1	R 6	R 2.5	R 2.5	R 2	R 2.5
7097 (*gsh1*Δ)	γ-Glutamyl cysteine synthetase	S 0.5	R 6	R 2	R 1	R 1	R 1
1740 (*gsh2*Δ)	Glutathione synthetase	S 1	R 6	R 2.5	R 1.5	R 2	R 3
6913 (*sod1*Δ)	Cu, Zn Superoxide dismutase	S 0	R 1.5	S0	R 1	R 0.5	S 0
6605 (*sod2*Δ)	Mn Superoxide dismutase	S 2	R 6	R 3.5	S2	S 2	R 3.5
2724 (*hog1*Δ)	MAPK	S 3	R 6	R 6	R 5	R 4.5	S 3
6116 (*sho1*Δ)	Transmembrane osmosensor	S 3	R 6	R 6	R 5.5	R 6	R 6
2720 (*ahp1*Δ)	Alkyl hydroperoxide reductase	S 3	R 6	R 6	R 6	R 6	R 6
4530 (*rad54*Δ)	DNA-dependent ATPase	S 2	R 6	R 5	R 2.5	R 3.5	R 3
775 (*sgs1*Δ)	ATP-dependent DNA helicase	S 2.5	R 6	R 6	R 4.5	R 6	R 6
R (%)			100	87	83	87	83
Mean R			5.6	4.2	3.5	3.8	3.8

[a]Numbers correspond to record-number for the respective deletion mutant

Table 2 In silico reconstruction of orthologous genes identified in the MAP kinase pathways and antioxidative stress response systems of *S. cerevisiae* and *Aspergillus flavus*. Genes of known function and identity from the annotated genome of *S. cerevisiae* were compared to expressed sequence tags (EST) from an EST library for *A. flavus*. Orthology was based on a BLAST search using amino acid sequences when an e-value <1 was recorded (see text)

S. cerevisiae gene definition		GenBank accession GI (Yeast)	*A. flavus* EST		*S. cerevisiae*/*A. flavus* similarity alignment			
Gene[a]	Function		ID	Length (bp)	Length (aa)	e-value	Homology	
							Identity	Similarity
Gene regulation								
569 (*YAP1*)	jun-like transcription factor	854483	TC4893	765	650	4E−11	31/59 (52%)	44/59 (74%)
			TC6037	761	650	2E−06	37/116 (31%)	49/116 (42%)
7117 (*MSN2*)	Transcription factor (Zinc finger)	695717	NAFAE55TH	701	704	4E−16	33/62 (53%)	45/62 (72%)
4911 (*MSN4*)	Transcription factor	486083	NAFAE55TH	701	630	3E−17	41/76 (53%)	49/76 (64%)
6957 (*HOT1*)	Transcription factor RNA polymerase II	854442	TC5612	677	719	3.6E+00	23/80 (28%)	43/80 (53%)
2045 (*SKO1*)	Transcription factor RNA polymerase II	1302140	NAFCN60TV	649	647	0.29	12/14 (85%)	14/14 (100%)
1983 (*URE2*)	Glutathione transferase-transporter regulator	1302253	TC5328	726	354	8E−27	74/225 (32%)	108/225 (48%)
Transporter/Assembly protein								
3143 (*FLR1*)	Multidrug transporter	536200	TC6573	850	548	2E−27	70/228 (30%)	116/228 (50%)
			NAFCX67TV	601	548	6E−22	56/160 (35%)	85/160 (53%)
5933 (*YOR1*)	ABC transporter	1323513	TC7882	978	1,477	5E−65	119/251 (47%)	162/251 (64%)
			TC5934	3,924	1,477	2E−43	218/915 (23%)	384/915 (41%)
			TC6058	1,121	1,477	1E−31	84/302 (27%)	150/302 (49%)
4969 (*VPH2*)	Assembly of vacuolar H(+)ATPase	486199	NAFCV74TV	803	215	0.84	15/40 (37%)	21/40 (52%)
Signal transduction								
6116 (*SHO1*)	Transmembrane osmosensor	603357	TC7445	648	367	7E−04	15/42 (35%)	25/42 (59%)
22306 (*SLN1*)	Histidine kinase osmosensor	6322044	NAFCM22TV	522	1,220	7E−34	65/122 (53%)	99/122 (81%)
			TC5569	613	1,220	2E−13	53/152 (34%)	86/152 (56%)
3439 (*STE50*)	Protein kinase regulator	5332	TC4957	685	346	0.92	20/62 (32%)	29/62 (46%)
956 (*STE20*)	Protein Ser/Thr kinase	508679	TC6393	652	939	1E−25	66/203 (32%)	113/203 (55%)
			TC4614	1,346	939	2E−17	63/208 (30%)	113/208 (54%)
23932 (*YPD1*)	Transferase activity	1431398	TC7659	424	167	1E−12	31/60 (51%)	45/60 (75%)
1561 (*SSK1*)	Two-component response regulator activity	1360296	TC5569	613	712	7E−14	48/165 (29%)	85/165 (51%)
			NAFCZ83TV	548	712	1E−07	30/116 (25%)	55/116 (47%)
			NAFCM22TV	522	712	4E−07	44/138 (31%)	69/138 (50%)
			NAFCH11TV	725	712	1E−04	17/58 (29%)	36/58 (62%)
2464 (*PTP2*)	Protein tyrosine phosphatase	1420487	TC7994	678	750	6E−09	38/95 (40%)	51/95 (53%)
215 (*PTP3*)	Protein tyrosine phosphatase	603312	TC7994	678	928	3E−06	26/70 (37%)	39/70 (55%)
2724 (*HOG1*)	Mitogen-activated protein kinase (MAPK)	1360508	TC4614	1,346	435	E−174	287/341 (84%)	311/341 (91%)
			NAGAA58TV	419	435	3E−59	101/139 (72%)	120/139 (86%)
			TC8338	671	435	6E−56	108/218 (49%)	147/218 (67%)
7101 (*HOG4*)	MAPK kinase (MAPKK)	1008328	TC6393	652	668	7E−16	59/189 (31%)	94/189 (49%)
			TC4614	1,346	668	3E−10	66/283 (23%)	121/283 (42%)
			TC7441	694	668	5E−10	36/90 (40%)	51/90 (56%)

Table 2 (continued)

S. cerevisiae gene definition		GenBank accession GI (Yeast)	*A. flavus* EST		*S. cerevisiae*/*A. flavus* similarity alignment			
Gene[a]	Function		ID	Length (bp)	Length (aa)	e-value	Homology	
							Identity	Similarity
7195 (*SSK22*)	MAPKK kinase (MAPKKK)	1907212	TC6393	652	1,331	7E−34	86/225 (38%)	119/225 (52%)
			TC4614	1,346	1,331	7E−18	83/310 (26%)	134/310 (43%)
			TC8338	671	1,331	3E−16	62/232 (26%)	115/232 (49%)
5406 (*SSK2*)	MAPKK kinase (MAPKKK)	1302527	TC6393	652	1,579	5E−32	85/237 (35%)	122/237 (51%)
			TC8338	671	1,579	4E−17	65/241 (26%)	116/241 (48%)
			TC4614	1,346	1,579	2E−16	85/325 (26%)	138/325 (42%)
5271 (*STE11*)	MAPKK kinase (MAPKKK)	4554	TC6393	652	717	2E−43	85/187 (45%)	125/187 (66%)
			NAGAA58TV	419	717	5E−16	42/111 (37%)	62/111 (55%)
			TC4614	1,346	717	2E−15	49/156 (31%)	78/156 (50%)
			TC8338	671	717	1E−13	57/244 (23%)	106/244 (43%)
Antioxidation								
4718 (*CTT1*)	Catalase T	1323129	TC7576	581	573	2E−05	40/158 (25%)	66/158 (41%)
3615 (*CTA1*)	Catalase A	1136211	TC7576	581	515	1E−13	44/143 (30%)	70/143 (48%)
2737 (*GLR1*)	Glutathione oxidoreductase	1151235	TC7940	733	483	7E−69	124/218 (56%)	166/218 (76%)
829 (*OSR1*)	Glutathione metabolism	736309	TC6854	864	442	3E−29	63/164 (38%)	91/164 (55%)
			NAFFN33TV	755	442	5E−16	41/133 (30%)	63/133 (47%)
24190 (*TRR1*)	Thioredoxin reductase	849175	TC5595	907	319	0.099	14/39 (35%)	20/39 (51%)
1934 (*TRR2*)	Thioredoxin reductase	529123	TC7940	733	342	0.41	23/75 (30%)	38/75 (50%)
545 (*TSA1*)	Thioredoxin peroxidase	575691	NAGAY31TV	831	196	7E−71	126/193 (65%)	150/193 (77%)
			NAFBN21TV	758	196	4E−17	58/194 (29%)	93/194 (47%)
			NAGAG45TV	610	196	3E−12	38/108 (35%)	58/108 (53%)
6681 (*GRX1*)	Glutaredoxin	5328	TC7366	528	110	6E−16	37/78 (47%)	54/78 (69%)
4347 (*GRX2*)	Glutaredoxin	927781	TC7366	528	143	1E−20	53/108 (49%)	70/108 (64%)
2654 (*TRX1*)	Thioredoxin	1360373	TC4733	636	103	2E−21	44/89 (49%)	62/89 (69%)
			NAGER47TV	567	103	7E−20	41/87 (47%)	60/87 (68%)
			TC6576	670	103	4E−18	38/82 (46%)	54/82 (65%)
4839 (*TRX2*)	Thioredoxin	1323375	TC4733	636	104	4E−25	47/103 (45%)	70/103 (67%)
			NAGER47TV	567	104	3E−22	40/101 (39%)	68/101 (67%)
			TC6576	670	104	3E−18	36/88 (40%)	58/88 (65%)
7097 (*GSH1*)	γ−Glutamyl cysteine synthetase	1008282	NAFDF35TV	637	678	7E−17	61/192 (31%)	92/192 (47%)
1740 (*GSH2*)	Glutathione synthetase	1419854	NAFFI48TV	745	491	2E−33	87/228 (38%)	123/228 (53%)
6913 (*SOD1*)	Cu, Zn Superoxide dismutase	1015812	TC6513	721	154	1E−57	103/152 (67%)	120/152 (78%)
6605 (*SOD2*)	Mn Superoxide dismutase	500704	TC6319	863	233	2E−68	128/226 (56%)	155/226 (68%)
2720 (*AHP1*)	Alkyl hydroperoxide reductase	1360500	TC5282	817	176	1E−25	69/161 (42%)	97/161 (60%)
			TC6625	700	176	5E−20	62/168 (36%)	93/168 (55%)
			TC8233	637	176	8E−09	26/52 (50%)	36/52 (69%)
DNA damage control								

Table 2 (continued)

S. cerevisiae gene definition		GenBank accession	*A. flavus* EST		*S. cerevisiae*/*A. flavus* similarity alignment			
Gene[a]	Function	GI (Yeast)	ID	Length (bp)	Length (aa)	e-value	Homology	
							Identity	Similarity
4530 (*RAD54*)	DNA-dependent ATPase	1322760	NAGCQ07TV	673	898	8E−61	123/224 (54%)	155/224 (69%)
			NAFEY35TV	681	898	2E−21	62/217 (28%)	102/217 (47%)
775 (*SGS1*)	ATP-dependent DNA helicase	349194	TC6652	688	1,447	2E−27	58/80 (72%)	66/80 (82%)
Other enzymes								
25391 (*ACC1*)	Acetyl CoA carboxylase	1302498	TC5329	839	2,233	7E−16	52/175 (29%)	80/175 (45%)
3718 (*GPD1*)	Glycerol-3-phosphate dehydrogenase	1430995	TC7617	856	391	1E−54	68/139 (48%)	87/139 (62%)
			TC8237	543	391	5E−46	86/159 (54%)	119/159 (74%)
199 (*HOR2*)	Glycerol-1-phosphatase	603298	TC9399	806	250	2E−26	67/198 (33%)	96/198 (48%)

[a]Numbers correspond to record-number for each deletion mutant

to 2.5 mM H_2O_2 and 0.4% of the respective antioxidant compound or 1 mM glutathione as a control antioxidant. Cell growth was monitored at 30°C for 7 days. Compounds were considered to have antioxidative activity if cell growth improved compared to cohorts exposed to H_2O_2 without the test compound. Cell growth was scored based on the serial dilution at which cell growth was no longer visible compared to control cohorts not exposed to H_2O_2 (e.g., numerical scoring was as follows: 6—colonies were visible in all dilutions, 0—no colonies were visible in any dilution, 1—only the undiluted colony was visible, 2—the undiluted and 10-fold diluted colonies were visible, etc., see Table 1).

Antiaflatoxigenic assays

Measurement of antiaflatoxigenic activities followed Mahoney and Molyneux (2004): gallic, tannic and caffeic acids, and methyl gallate (Sigma) were individually dissolved in distilled water, filter-sterilized, incorporated into VMN at 0 (control), 0.05, 0.1, 0.2, or 0.4% (w/v), inoculated with ~200 spores of *A. flavus* NRRL 25347 and incubated at 28°C. After 12 days, fungal mats were removed, extracted with methanol (50 ml), an aliquot (1 ml) was dried under N_2 and the residue derivatized with trifluoroacetic acid. Aflatoxin B_1 (AFB1) was quantified by reverse-phase HPLC (only trace levels of aflatoxin B_2 were detected).

In silico identification of *A. flavus* genes

An amino acid based BLAST search of the EST database of *A. flavus* NRRL 3357 (Yu et al. 2004b; http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=a_flavus) identified orthologs of yeast genes in stress response/tolerance and signal transduction pathways when an alignment value was $e<1$. Details concerning yeast genes and deletion mutants are available from the Stanford Genome Technology Center (http://www-sequence.stanford.edu/) and NCBI DBEST (http://www.ncbi.nlm.nih.gov/dbEST/index.html).

Functional complementation assays

Functional complementation of the superoxide dismutase 2 gene of *A. flavus* (*sodA*) was tested in yeast *sod2*Δ. The reverse-transcribed first strand cDNA of *sodA* was synthesized by cultivating *A. flavus* strain NRRL 3357 in YES (2% yeast extract, 6% sucrose, pH 5.8) at 28°C overnight, isolating total RNA (RNeasy Plant Mini Kit, Qiagen, Valencia, Calif.) and using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Beverly, Mass.) and *sodA*-C (tctagatctagaCCGAGGCGTATCCAATTGCAT TAGGGCTAC; lower case includes *Xba*I site) as the primer. The *sodA* coding region was obtained by RT-PCR of the cDNA template [95°C (2 min) 1 cycle; 95°C (30 s), 55°C (30 s), 68°C (1.5 min) 25 cycles, 68°C (15 min) 1

cycle; enzyme: Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen)] and *sodA*-N (ggatcc**g**gat**cc***ATG*AA CTCAATTCCATTGATACATAAAAATC[ATG initiation codon in italics; bold letters: Kozak sequence (Kozak 1987); lower case: two *Bam*HI sites] and *sodA*-C as primers. The PCR product was digested with *Bam*HI and *Xba*I, purified (QIAEX II gel extraction kit, Qiagen) and subcloned into the yeast expression vector pYES2 (Invitrogen), which has a *GAL1* promotor. Genomic *sodA* was isolated by PCR of genomic DNA from *A. flavus* NRRL 3357 [see Skory et al. (1992) for genomic DNA isolation] using primers *sodA*-N and *sodA*-C. This DNA was sequenced using primers *sodA*-N, *sodA*-C, *sodA*-N1 (ATCAACTTC CACGGTGGTGG) and *sodA*-C1 (TCGTCGATGGCC TTGGCCAA) (Davis Sequencing, Davis, Calif.). The *sodA* gene sequence was then deposited with GenBank (accession no. AY585205). To test functional complementation, yeast *sod2*Δ with empty pYES2 (negative control), wild-type with empty pYES2 (positive control), wild-type and *sod2*Δ with pYES2 carrying the *sodA* coding region, and wild-type and *sod2*Δ strains without vector were cultured in a raffinose medium (0.67% yeast nitrogen base without amino acids, 2% raffinose, 0.03 mg ml^{-1} amino acids) overnight at 30°C. Two independent transfectants for each negative and positive control, and functional complement were included. Cells of each strain-vector combination were serially diluted in liquid raffinose medium and spotted, as described above, on SGAL plates incorporating H_2O_2 (1.0, 1.5, 2.0, 2.5 or 3.0 mM). The *sodA* gene was considered a functional ortholog if cell growth was identical to the positive control or better than negative controls.

Results

Alleviation of oxidative stress in wild-type yeast

Exposure of WT yeast to 3.3 mM H_2O_2 significantly inhibited cell growth, with only the colony arising from undiluted cells visible in the assay (Fig. 1). Test compounds were not toxic at 0.4% (w/v) as all colonies arising from all dilutions were visible. The toxicity of H_2O_2 was significantly alleviated by treatment with tannic, gallic and caffeic acids, and methyl gallate, with alleviation increasing with increased addition of the compounds from 0.05 to 0.4% (Fig. 1). Methyl gallate and caffeic acid showed highest alleviation, with significant alleviation even at 0.05% and almost full alleviation at 0.1–0.2%. Gallic acid showed greater activity than tannic acid, with almost full alleviation when added at 0.4%. Thus, all compounds were considered as antioxidants.

Effects of oxidative stress and antioxidants on deletion mutants

Oxidative stress and its alleviation by antioxidants were examined in 22 different deletion mutants to identify yeast antioxidative stress response genes using the identified antioxidants and glutathione, an antioxidant control. Deletion mutants examined included strains defective in gene regulation (*yap1*Δ), DNA damage control (*rad54*Δ, *sgs1*Δ), signal transduction (*hog1*Δ, *sho1*Δ), oxidative stress response (*ctt1*Δ, *cta1*Δ, *glr1*Δ, *osr1*Δ, *trr1*Δ, *trr2*Δ, *tsa1*Δ, *grx1*Δ, *grx2*Δ, *trx1*Δ, *trx2*Δ, *gsh1*Δ, *gsh2*Δ, *sod1*Δ, *sod2*Δ), and a vacuolar H (+) ATPase assembly protein (*vph2*Δ). Examples of visual bioassays of deletion mutants exposed to 2.5 mM H_2O_2 and antioxidant compounds are shown in Fig. 2 with responses of all deletion mutants tabulated in Table 1. Hydrogen peroxide was moderately to severely lethal to all yeast strains, but all antioxidants tested fully alleviated oxidative stress in WT yeast (Table 1). Alleviation varied among the deletion mutants depending on the deleted gene and/or antioxidant. For example, oxidative stress was almost fully alleviated by glutathione in all strains with the exception of *yap1*Δ and *sod1*Δ. These latter mutants did not recover well using any of the compounds tested here, indicating high sensitivity to oxidative stress. For example, results with *yap1*Δ are consistent with prior work showing that Yap1 induces

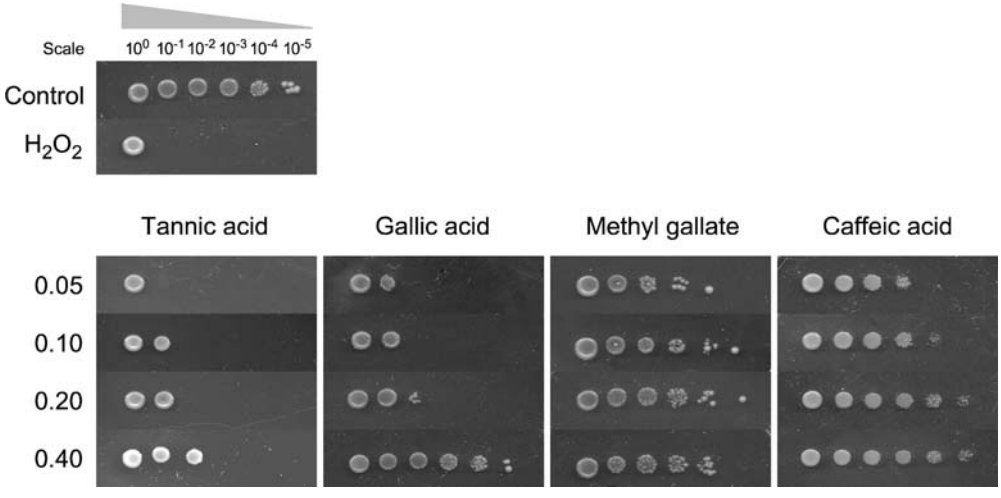

Fig. 1 Oxidative stress bioassays on wild-type yeast. *Upper panel* Control conditions without H_2O_2 or test compounds and treatment with H_2O_2 (3.3 mM) only. The scale represents serial dilutions of approximately 10^6 cells after a 7-day incubation at 30°C. *Lower panel* Treatment with H_2O_2 and 0.05, 0.10, 0.20 and 0.40% (w/v) of tannic, gallic and caffeic acids, or methyl gallate

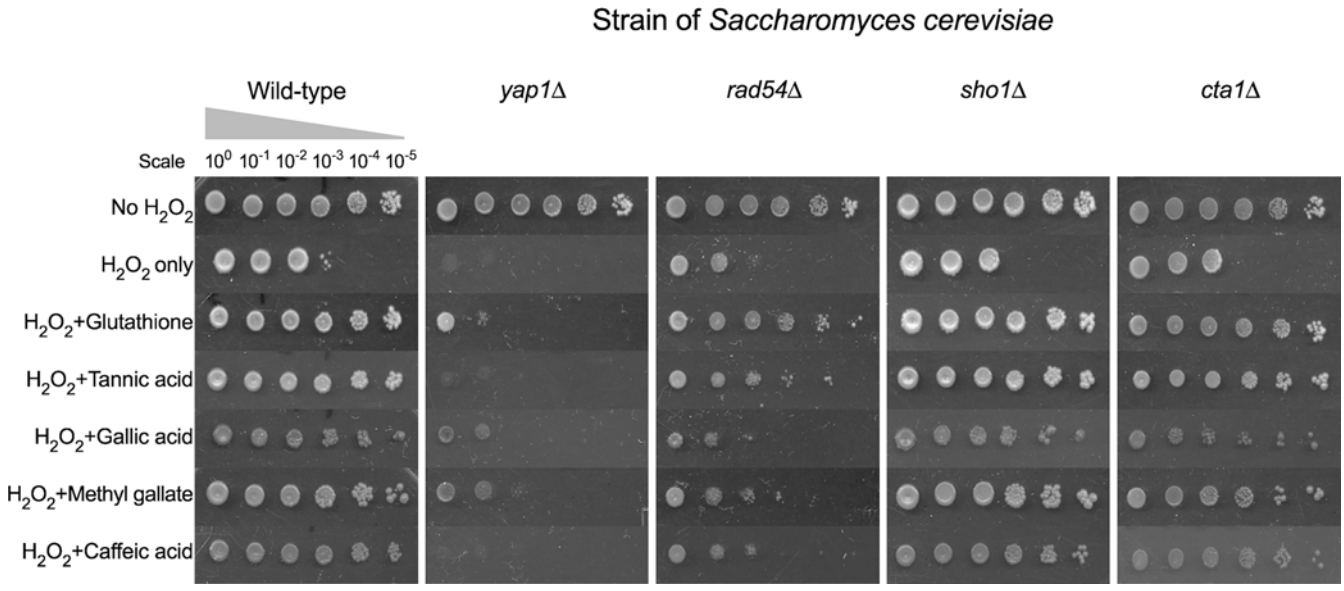

Fig. 2 Oxidative stress bioassays of yeast deletion mutants. Examples include wild-type (WT) and yeast deletion mutants of *yap1*Δ, *rad54*Δ, *sho1*Δ and *cta1*Δ. Controls received no treatment. Treatments included H_2O_2 (2.5 mM) and 0.4% tannic, gallic or caffeic acids, or methyl gallate. Glutathione (1 mM) served as a positive antioxidant control. The scale represents serial dilutions of approximately 10^6 cells after a 7-day incubation at 30°C. See Table 1 for all results

downstream expression of *GLR1*, *TRR1*, *TRX* and *GSH1* (Fernandes et al. 1997; Lee et al. 1999), individual deletion mutants of which recovered when treated with antioxidants. Other mutants showing only moderate-to-low recovery ($R \leq 2.5$) with treatment included *glr1*Δ, *tsa1*Δ, *vph2*Δ, *trx2*Δ and *gsh1*Δ. The mutant of *SHO1*, which encodes a transmembrane osmosensor, recovered almost fully when treated with any of the antioxidants. Tannic and caffeic acids alleviated toxicity at different levels in all strains except *yap1*Δ, *vph2*Δ, and *sod1*Δ and caffeic acid did not alleviate stress at any level in *hog1*Δ. Gallic acid alleviated toxicity in all strains except *glr1*Δ, *tsa1*Δ, *vph2*Δ and *sod2*Δ, while methyl gallate alleviated toxicity in all strains except *glr1*Δ, *vph2*Δ and *sod2*Δ. Thus, the test compounds possess activity with respect to oxidative stress genes, methyl gallate appearing to have the broadest activity.

Antiaflatoxigenesis by caffeic acid and components of hydrolysable tannins

Caffeic acid was a potent antiaflatoxigenic agent—equal to that of the constituents of hydrolysable tannins—completely inhibiting aflatoxigenesis at a concentration of 0.1% (w/v) (data not shown), similar to tannic and gallic acids and methyl gallate. None of these compounds had a visible effect on fungal development at this concentration. The antioxidant and antiaflatoxigenic activities of these compounds suggested a relationship may exist between oxidative stress and aflatoxin biosynthesis in *A. flavus*.

In silico identification of MAP kinase pathway and antioxidative stress genes

A total of 43 orthologs of yeast mitogen-activated protein kinase (MAPK) pathway, antioxidative stress response and DNA damage control genes were identified using the *A. flavus* EST database (Table 2). Amino acid sequence similarity varied between orthologs, depending on the type of gene. For example, three orthologs of yeast *HOG1*, a MAPK cellular signal transduction gene, were identified in *A. flavus*, ranging in similarity between 67 and 91% compared to the yeast ortholog. Several MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) gene families, acting upstream of MAPK, were also identified, indicating that *A. flavus* possesses several signal transduction pathways to process developmental and/or environmental elicitors. Several orthologs of antioxidant enzyme/proteins, e.g., glutathione, thioredoxin and alkyl hydroperoxide reductase, were identified with high degrees of similarity (>60%) to their counterparts in yeast. The existence of these multiple gene families indicates that an elaborate oxidative stress response is available to *A. flavus*. Orthologs involved in gene regulation, such as transcription factors (e.g., *YAP1*) had relatively lower similarities (~40–70%) compared with signal transduction and antioxidative stress genes. Limited regions of similarity, however, indicated possible active domains. In summary, a number of stress response pathway genes were identified in the *A. flavus* EST database for future functional analysis to examine the potential relationship between oxidative stress and aflatoxin biosynthesis.

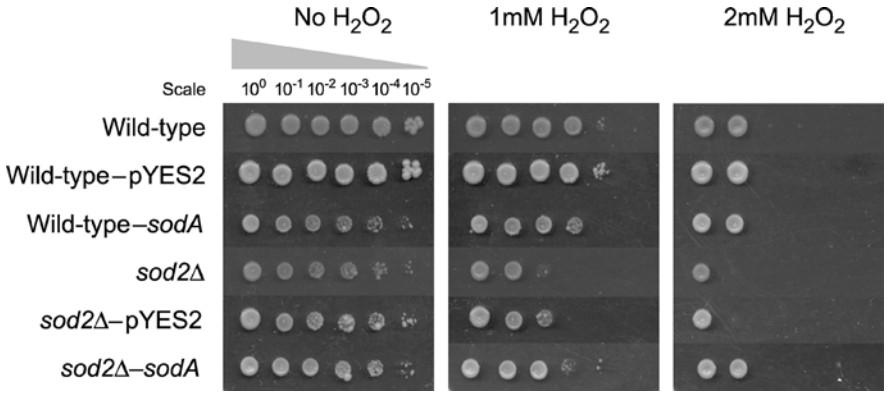

Fig. 3 Functional complementation of yeast mutant *sod2*Δ with an *A. flavus* superoxide dismutase gene (*sodA*), under oxidative stress at 1.0 and 2.0 mM H_2O_2. Oxidative stress (H_2O_2 1.0, 1.5, 2.0, 2.5 and 3.0 mM) was induced in untransformed WT and the *sod2*Δ deletion mutant of *S. cerevisiae* transformed with either empty vector (pYES2) or vector with *SOD2* from *A. flavus* (*sodA*). Levels of survival of the *sod2*Δ strain were ~100-fold and at least 10-fold greater at 1.0 and 2.0 mM H_2O_2 treatments, respectively, if transformed with *sodA*, and were equivalent to WT. WT and *sod2*Δ strains transformed with empty vector responded similarly as their respective untransformed counterparts. The scale represents serial dilutions of approximately 10^6 cells after a 7-day incubation at 30°C

Functional complementation of *S. cerevisiae sod2* Δ with *A. flavus sodA*

Complementation analysis using the Mn superoxide dismutase (Mn-SOD) ortholog of *A. flavus* (*sodA*) in the yeast *sod2*Δ mutant was performed to demonstrate the utility of high-throughput yeast deletion mutant bioassays as a model for *A. flavus* stress response genes. A BLAST search of the NCBI database revealed that SodA has a high degree of amino acid similarity (~50% identity based on BLOSUM 62 alignments) to orthologs from a broad phylogenetic spectrum of fungi (e.g., *A. nidulans* 82%, *Neurospora crassa* 63%, *Magnaporthe grisea* 63%, and *S. cerevisiae* 53%, etc.) and other taxa (chicken, mouse and human). Highest similarity was almost entirely within highly conserved – and C- terminal domains, also in prokaryotes (Marchler-Bauer et al. 2003), indicating the universally important role of Sod2 in the oxidative stress responses of all prokaryotes and eukaryotes.

Growth of either WT or *sod2*Δ yeasts transformed with an empty pYES vector was no different from the corresponding untransformed cohorts under control conditions (Fig. 3). A slightly slower growth rate was observed for the WT containing *sodA* and the *sod2*Δ mutant containing either an empty or *sodA*-containing vector. The *sod2*Δ strain with the *sodA*-containing vector showed practically the same levels of survival as the WT with the empty or *sodA*-containing vector at all H_2O_2 exposure concentrations (Fig. 3). The *sod2*Δ strain with an empty vector was ~100-fold and 10-fold more sensitive to H_2O_2 at the 1.0 and 2.0 mM treatments, respectively, than *sod2*Δ with *sodA*. Hence, the poorer survival of the yeast *sod2*Δ mutant under oxidative stress, and improved survival in the presence of *A. flavus sodA* indicates that *sodA* is the functional ortholog of the yeast *SOD2* gene.

Discussion

Antioxidative compounds can be used to examine the functional genomics of fungal oxidative stress genes. In this report, we present a novel assay system using specific yeast deletion mutants and gene complementation for high-throughput evaluation of gene function. We identified at least 43 genes in *A. flavus* associated with stress responses possibly linked to aflatoxigenesis and demonstrate the high-throughput technique using a mitochondrial antioxidative stress gene. Yeast strains lacking mitochondrial function are sensitive to oxidative stress (Grant et al. 1997) and Mn-SOD (Sod2), located in the mitochondrial matrix, detoxifies superoxide radicals generated by the mitochondrial respiratory chain (Zelko et al. 2002). The fact that *sodA* of *A. flavus* functionally complements the yeast mitochondrial SOD mutant demonstrates the potential for *S. cerevisiae* to serve as a model system for further high-throughput analysis of other *A. flavus* genes. Yeast *AHP1*, a gene involved in redox homeostasis, has also been functionally complemented in a yeast *ahp1*Δ mutant by the *A. flavus* ortholog (G. Payne, personal communication). Moreover, deletion mutants common to our study and those examined by Tucker and Fields (2004) did not differ qualitatively in their sensitivities to oxidative stress, further verifying the utility of our high-throughput bioassay system.

In this study we also showed that certain antioxidative compounds inhibited aflatoxin biosynthesis. Previously, it was shown that aflatoxin production by *A. parasiticus* was triggered by increased oxidative stress (Jayashree and Subramanyam 2000), and that antioxidants, such as eugenol (Jayashree and Subramanyam 1999) and hydrolysable tannins (Mahoney and Molyneux 2004), reduced aflatoxin production. Our study found a number of antiaflatoxigenic, antioxidative compounds that alleviate ox-

idative stress in yeast strains lacking antioxidative stress genes, further indicating an association between oxidative stress and induction of aflatoxin biosynthesis. Infection of plant cells by pathogens such as *A. flavus* can induce rapid generation of reactive oxygen species (ROS) (Levine et al. 1994) and production of hydrogen peroxide (Bolwell 1999) during plant defense responses. Other sources of oxidative stress associated with increased aflatoxin contamination include high temperature, drought and UV (Estruch 2000; Wang et al. 2003). The functional genomic tools of our study should greatly facilitate our understanding of how oxidative stress might trigger aflatoxin biosynthesis.

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